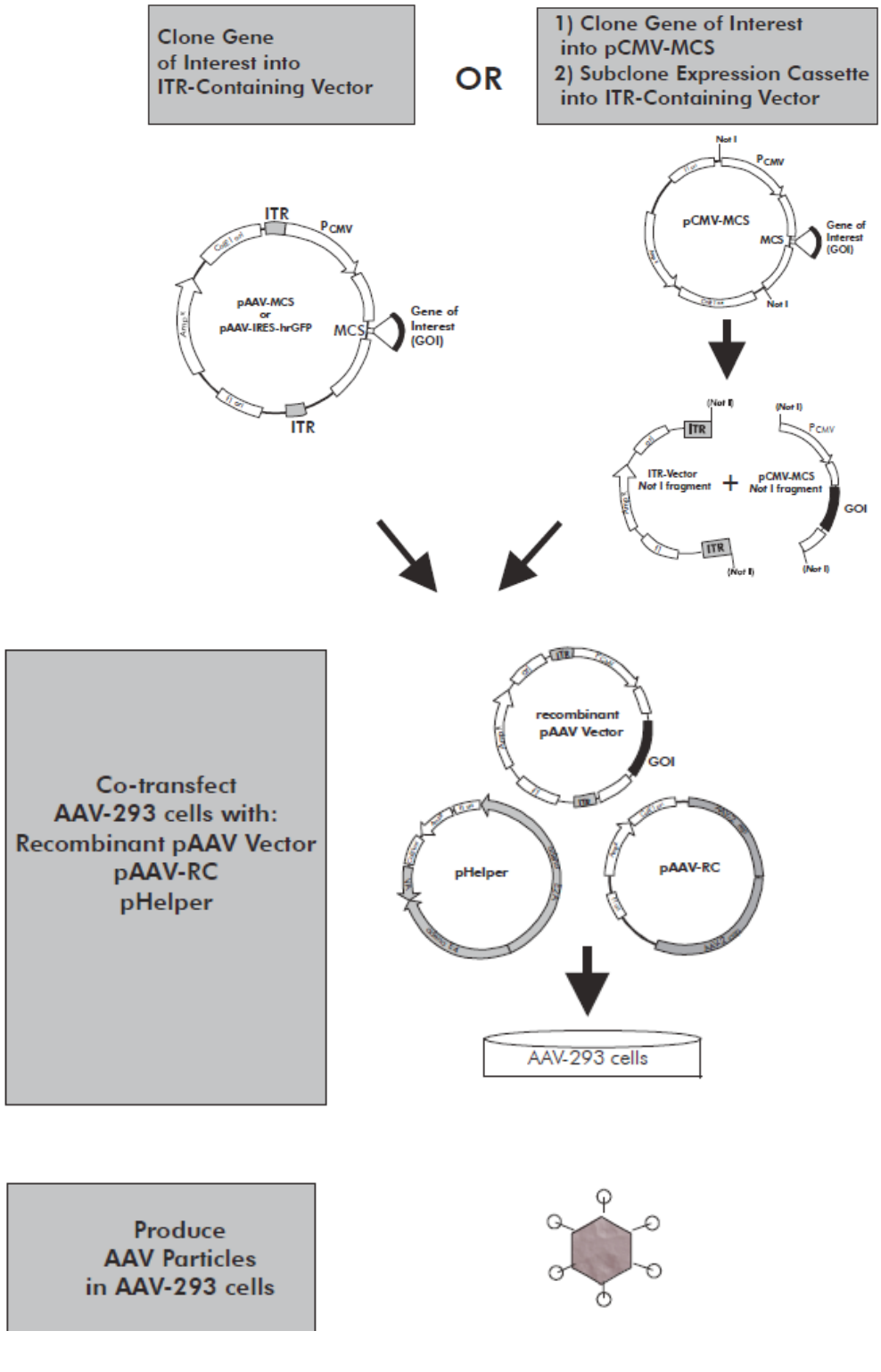
Clone Gene of Interest into ITR-Containing Vector
OR
1) Clone Gene of Interest into pCMV-MCS
2) Subclone Expression Cassette into ITR-Containing Vector
ITR
PCMV
pAAV-MCS or pAAV-IRES-hrGFP
MCS
Gene of Interest (GOI)
ITR
Not I
PCMV
pCMV-MCS
MCS
Gene of Interest (GOI)
Not I
(Not I)
ITR
(Not I)
PCMV
ITR-Vector Not I fragment
+
pCMV-MCS Not I fragment
GOI
ITR
(Not I)
(Not I)
Co-transfect AAV-293 cells with: Recombinant pAAV Vector pAAV-RC pHelper
recombinant pAAV Vector
GOI
pHelper
pAAV-RC
AAV-293 cells
Produce AAV Particles in AAV-293 cells

AAV Production

Materials

- DNA samples
- DMEM (Biological industries), per 500 ml:
    - 10 ml 100x non-essential amino acids
    - 10 ml 100x sodium pyruvate
    - 10 ml L-glutamine
    - 5 ml penicillin/streptomycin
- 2.5 M $CaCl_2$ (filtered through 0.22 um sterile filters)
- 2x HeBS pH 7.05 [280 mM NaCl, 50 mM HEPES, 1.5 mM Na_2HPO_4, adjust pH with NaOH]. pH is absolutely crucial!!! Store aliquots at -20 °C. Once thawed, aliquots can be stored at 4 °C.
- sterile ddH2O
- sterile PBS
- Lysis buffer [150 mM NaCl, 50 mM Tris-HCl pH 8.5]
- Benzonase
- 0.45 um sterile filters
- 0.22 um sterile filters
- syringes (1, 2, 5, 10 ml)

Day 1 – cell culture (seed cells)

- Seed $2{\cdot}10^6$ HEK 293 cells per 15 cm plate in 20 ml DMEM. Prepare 2 plates for each vector transfection

1. Aspirate medium.
2. Add 3 ml trypsin, mix gently, and put into the incubator for 3 minutes
3. Add 7 ml of medium to detached cells for Trypsin inactivation.
4. Count the cells and seed $2{\cdot}10^6$ HEK 293 cells per 15 cm plate (2 plates per each vector transfection) in total volume of 20 ml. Mix gently
5. Place the cells in a 37°C incubator at 5% CO2.

Day 2 – transfection (calcium phosphate-based protocol)

1. 24 h post seeding, control cell confluent (optimal around 50%)
2. Bring $CaCl_2$ (2.5 M) to room temperature and pre-warmed HPSS at 37°C
3. pAAV expression plasmid for each plate- 12.5 µg (if we have 2 plates per virus= 25 µg). Helpers-
   12.5 µg of pAAV-RC (per plate)
   12.5 µg of pHelper (per plate)
4. In 50 ml falcon tube, add plasmids (pAAV expression plasmid + pAAV-RC+ pHelper), 500 µl of $CaCl_2$, water (sterile- open only in hood) up to 2 ml, and mix gently.
5. Add 2 ml HPSS- drip slowly!
6. Vortex the falcon. The liquid will become cloudy.
7. Add 2 ml from the transfection solution dropwise to each cell culture plate- Mix gently.
8. Place plates back to the incubator.

Day 3 – medium exchange

One day post transfection change medium with 20 ml of fresh complete medium

Day 5 – Preparing Viral Stocks

ALL THE WASTE – INTO A SPECIAL BAG – UV

1. Prewarm **lysis buffer**
2. Collect the cells from the plate, scrape the cells medium with a cell lifter, while holding the plate at an angle. Collect cells into 50 ml falcon tubes (2 plates into one tube).
3. Centrifuge for 15 min, at 120 g (=1050 rpm)
4. Discard supernatant
5. Add buffer lysis (0.5 ml per plate →2 plates- 1 ml) pipette well and transfer into fresh 15 ml tube
6. Add 1 ml buffer lysis to the tube and pipette
7. Subject the cell suspension to 3 rounds (10 min each) of freeze/thaw by alternating the tubes between the dry ice-ethanol bath and the 37°C water bath, vortexing briefly after each thaw.
   Add 0.75 µl of Benzonase (50 U) to each tube
8. Incubate for 1.5 hours at 37°C

9. Spin 3000g (rcf), 4°C, 15 min
10. Collect supernatant in a syringe and filter through a 0.45 μm filter into fresh 15 ml tubes
11. Spin again
12. Collect supernatant in a syringe and filter through a 0.45μm filter into 15 ml.
13. Transfer 700 μl from the virions to eppendorf (use tips with filter!!)
14. Store at 4°C sealed with parafilm

HPSS buffer preparation

(100ml)

1. Add 1.6 g of NaCl (280mM)
2. Add 0.027 g of $NaPO_4X2H_20$ (1.5mM)
3. Add 0.2 g of dextrose (12mM)
4. Add 1 g of HEPES (50mM)
5. Add up to 90ml ddH_20
6. Titration- Measure pH and add NaOH until pH is 7.05

Lysis buffer preparation

(500ml)

1. Add 4.38 g of NaCl (MW=58.44 g/mol) → 150 mM
2. Add 3.94 g of Tris-HCL (MW=157.6 g/ml) → 50 mM
3. In erlnmayer- add 400ml ddH_2O
4. Titration- Measure pH and add NaOH (1M or 0.5M) until pH is 8.5
5. Add the rest of the water- total 500ml
6. Transfer through filter in hood

- NaOH 1M – 40 gr into 1L

DMEM preparation

Materials:

- DMEM (500ml)
- Sodium pyruvate
- Penstrep (penicillin streptomycin)
- L- glutamine
- FBS

1. Thaw the materials
2. Transfer 50 ml of DMEM to 50 ml flacon
3. Add 50 ml of FBS
4. Add 5 ml penstrep
5. Add10 ml L-glutamine (vortex before adding)
6. Add 10 ml sodium pyruvate (vortex before adding)